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Complement Fixation Reactions With Sphaerophorus Necrophorus Antigens

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COMPLEMENT FIXATION REACTIONS WITH *SPHAEROPHORUS NECROPHORUS* ANTIGENS

By

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INTRODUCTION

The financial loss incurred by the condemnation of beef livers abscessed with Sphaerophorus necrophorus, has been described in many reports previous to this. Canada (4) reports that between two and twenty percent of all beef livers are condemned as a result of pathological conditions of the liver. Eighty five percent of all abscessed livers contain a pure culture of S. necrophorus. (13)

This organism seems to be present in the western and midwestern states, which seems logical because these are areas in the United States where the greatest number of cattle are grazed. It seems apparent that although the animal may contract S. necrophorus on the range the greatest incidence occurs in feedlot cattle. In connection with this another item of interest is that fattened cattle have a higher incidence of infection than non-fattened cattle. No definite explanation has been advanced for this to date. The transmission of this organism is not well understood.

Macroscopically, in fluid anaerobic culture media, the organisms usually appear to grow in a linear fashion from the surface of the medium downward to the butt of the tube. Some strains grow, dispersed throughout the medium, as spherical, yellowish colonies one-fourth centimeter in diameter. Microscopically the organisms appear as long filaments.

S. necrophorus is an extremely fragile organism. It requires a medium possessing an extremely low oxygen tension. Work is being done

at present concerning the nutritional requirements of the organism. Grant (10) has shown that a medium containing only casitone, glucose, sodium chloride and L-cystine yielded significant growth of the organism.

Although this organism rarely infects man it has been responsible for the deaths of several meat packers and inspectors. Smith (20) states that abscesses on the hands, lungs and liver have all been reported in man. He states too that the organism may cause septicimia in man. Beigelman (1) reports that the rate of infection is greatest in females. Young adults (16-30) are most often infected although all age groups are represented. It may be positively stated that S. necrophorus, in man, enters only impaired tissue. Healthy tissue appears resistant to the organism.

Although this organism is most commonly associated with beef liver necrosis it is also responsible for calf diphtheria. Here it grows in thick membranes lining the respiratory passages. It is also an organism responsible for foot rot in sheep. (20)

S. necrophorus is propagated in vivo in rabbits, Guinea pigs and mice. Smith (20) states that of the laboratory animals Guinea pigs are most resistant. He also makes clear the fact that the organism is almost always a lethal agent in mice and rabbits. Other authors substantiate this observation. (20, 13, 4)

Few authors (20, 2, 16) give reference to serological work dealing with this organism. Serological reactions of S. necrophorus constitute the body of this thesis. The serological method employed is the complement-fixation reaction.

REVIEW OF LITERATURE

The Armour packing plant in Huron, South Dakota reported on abscessed condition in the livers of 10.9 percent of 14,725 cattle slaughtered in 1958.

The organism that is primarily responsible for the abscessed condition of bovine livers is S. necrophorus. (13) Its classification, according to Bergey's manual (3) is as follows:

Order IV: Eubacteriales buchanan, 1917.
Family : Bacteroidaceae
Genus : Sphaerophorus
Species : necrophorus

The organism was first isolated and classified by Flugge in 1886, as Bacillus diphtheriae vitulorum. In the years following 1886 the organism has been reclassified several times. (3) In 1938 Prevot attached to the organism the binomial name Spherophorus necrophorus. Dcotor Robert S. Breed of Cornell University assisted by Doctor Heiner Hoffman of the college of Dentistry at New York University, in 1955, revised the spelling to Sphaerophorus necrophorus. (3)

Most strains of the organism show curved filaments up to 100 microns or longer but after long subculturing few filaments of over 10 microns are seen. After five to ten weeks of incubation all of the organisms gradually disappear until only amorphous material is left. Such cultures are found to be sterile upon subcultivation. (2) There is a certain amount of conjecture, by workers, concerning the formation of branched forms of the organism. Bergey's Manual of Determinative Bacteriology (3) points out that some authors have reported branched

forms of the organism.

Wilsom and Miles (22) claim that branching does not occur. Louis Smith (20) refers to W.B. Smith's work on the mode of reproduction, distinct from the usual mode of fission, of S. necropherous as observed by electron micrograph studies. With a pleomorphic strain that they examined at about nine hours of incubation, they found that the oval or irregular swellings which developed in the middle or near the ends of the cells increased in size until all of the cellular material was within the swelling or round body, except for a small tail which was a portion of the original rod. Some of these swellings became wholly round. By 24 hours of incubation the cultures consisted chiefly of round bodies. When such round bodies were transferred to new media some of them segmented into four or five bacillary cells of normal size. Others, however, sent out filaments at two to six points on the surface and these filaments then broke up into daughter cells by segmentation. Most strains of S. necrophorus lose the ability to form filaments after several transfers. This can be restored by cultivation on medium containing penicillin. (20) Nolechek (15) stated that the organism occurs most commonly in the filamentous form but older cultures, whether in animal tissue or artificial media, exhibit almost exclusive cocco-bacilli.

Klienberger-Nobel (12) is of the opinion that the L type of the organism may be regarded as a process of regeneration probably equivalent to a sexual process in higher organisms.

It has not been established conclusively that the organism is motile. The organism is Gram negative. (3) It can be made to exhibit

a beaded appearance by the use of a dilute carbol-fuchsin dye. (17) Canada (4) reports the appearance of granules or septae in the organism.

S. necropherous has been considered by all earlier workers, to require an anaerobic atmosphere for growth on solid media. A symbiotic relationship exists in media between S. necrophorus and Staphylococcus aureus. The latter is strongly aerobic. It uses the oxygen present thereby allowing the obligate anaerobe to thrive. (2) Grant (10) reports that the organism retains its viability upon repeated washing. There is a great deal of controversy concerning the in vitro cultivation of the organism. Wilson and Miles (22), Bergey's Manual (3) and Smith (19) list media with which they were successful in propagating the cells. On the other hand Grant (10) in over 200 trials, reports generally unsatisfactory results in the isolation of S. necrophorus. Several methods have been outlined for the procurement of the organism in pure culture. Schrivner and Lee (17) attempted to isolate the cells in pure culture by subcutaneous injection of purelent material into rabbits. They found that cultures from the subcutaneous lesions invariably showed growth of many contamination organisms, but not S. necrophorus. Heart blood injections from the infected animal into nutrient media, also yielded no growth of the organism. Canada (4), reports the isolation of the organism by the extraction of blood from an infected rabbit and its injection into fluid thioglycollate medium.

S. necrophorus is present in several pathological conditions in many species of animals. It is a rare, even exotic, cause of human disease. (1) It causes "calf diphtheria", in which it grows as a thick

membrane lining the respiratory passages. Flint and Jensen proved that the organism causes foot-rot in ungulates. (9) One of its principal manifestations is in the liver of cattle. Jensen and co-workers (11) believed that the organisms enter the liver by the portal blood stream via a lesion of the rumen. Fitch (8) writes that S. necrophorus causes five diseases in swine: necrotic stomatitis, necrotic rhinitis, necrotic gastrinitis and enteritis, necrotic dermatitis, and necrotic pneumonia. Wilson and Miles (22) claim that the organism is not pathogenic for guinea pigs, dogs, cats, pigeons and hens.

Speaking of the histology of the lesions produced by S. necrophorus Beveridge (2) describes three general conditions: (a) Chronic, (b) Acute, (c) Peracute. The chronic type of infection is most often associated with the abscessed liver condition of cattle. It is characterized by having a thick band of fibrous tissue surrounding a mass of pus cells and necrotic material in which comparatively few organisms are seen. Those found are from two to ten microns long. In chronic stages of abscess formation and throughout the entire course of the disease the animals are not clinically sick and consequently continue to eat and gain weight. (11) The acute form of the infection is found in cases of "jaw disease". Jensen, Flint and Griner (11) write that in the early stages of abscess formation the animals are sick and may die. Rabbits dying of experimental inoculation exhibit the peracute form of infection.

According to Madin (13) S. necrophorus is the principal bacterial agent responsible for the pathology produced in bovine liver abscesses. S. necrophorus has not been cultured from healthy livers so is assumed

not to be present there.

Shaw (18) feels that agglutination tests are not an aid to the diagnosis of an infection of S. necrophorus. Strains of the organism are antigenically heterogenous (5) although a few strains share antigens and cross agglutinate. Strains from liver abscesses in cattle tend to be more uniform than do strains from infections in humans. (20) Titers of 1:800 are found in the blood of normal ungulates. (22)

Smith (20) writes that attempts at animal immunization have yielded an immunity of low order or none at all. Beveridge (2) was unable to demonstrate immunity. A factor which may have operated against successful immunization was the production of a subcutaneous localized necrotic abscess by the vaccine. This persisted for over a month so that the bulk of the antigen remained localized and had little opportunity of stimulating antibody response. (2) Elder, et al. (7) report that they have succeeded in preparing an antiserum which would protect rabbits against experimental inoculations of the organism.

Both soluble exotoxins and endotoxins have been reported. (2, 14, 5, 22) The exotoxins produce an edema when inoculated intradermally into rabbits. The ability to produce lesions in animal tissue is largely due to the production of a necrotizing endotoxin found in the cells of the organism.

Flinto (9) has found that few liver abscesses occur in cattle when fed 70 milligrams of Aureomycin per animal per day. Shaw (18) states that there is a possibility that x-ray treatment may be beneficial in the treatment of both visceral and cutaneous lesions.

PROCEDURES, RESULTS AND DISCUSSION

Having conferred with several meat packers and by reference to certain journal articles it became apparent that there was no readily available method of diagnosing, by serological methods, beef liver abscesses resulting from infection with S. necrophorus.

In a vein of applied research an effort was made to adapt a modification of the complement fixation reaction to the diagnosis of this bovine infection. An exploration was made concerning the possibility of developing a common antigen with which all positive antisera would react. An attempt was made to determine whether the antigenicity was a function of the cell wall or the somatic portion of the cell.

In performing the tests, antisera were obtained from the blood of fourteen animals which were observed to have livers infected, to varying degrees, with S. necrophorus. It was believed that a suitable antibody titer had been built up as a result of the organism's having been present in the animal's hepatic lobes. Pooled serum from three animals whose livers had appeared normal was used as a control serum. The M-2 serum was that which was derived from a rabbit in which an antibody titer had been built up by a series of intervenous M-2 antigen injections. The M-2 antigen consisted of an organism which, by penicillin attenuation, had been adapted to produce abscessed livers in mice. Antiserum 11366 was that of a rabbit which had received a series of intravenous injections of killed S. necrophorus strain 11366 obtained from the American Type Culture Collection. It was employed to serve as a reference antigen for workers, who, in later serological

work, with this organism, would desire to compare their work with this.

Eight types of antigen were prepared as described below. Unless designated, the letters attached to the antigens and antisera would have no significance except that they provide identification.

Physiological saline was prepared by dissolving 8.5 grams of chemically pure sodium chloride in a liter of distilled water. The solution was buffered to pH 7.0 with molar solutions of K_2HPO_4 and KH_2PO_4. This solution was sterilized in an autoclave for 15 minutes at 121°C. and used, in all of the subsequent dilutions, as the suspending medium and electrolyte.

The titration of complement was effected by placing into each of nine tubes a 1:10 dilution of complement. The amount placed in each tube varied from 0.10 cubic centimeter in tube one to 0.60 cubic centimeter in tube nine. The aliquot placed in each successive tube increased by an increment of 0.05 cubic centimeter. Each tube was then diluted, with saline solution, to a volume of 1.50 to 1.55 cubic centimeters. The solutions were mixed and placed in a 37°C. constant temperature water bath for one hour. Into each of the tubes was placed 0.5 cubic centimeter of a 1:100 dilution of antimouse amboceptor which by titration was found to be equivalent to two amboceptor units. Also placed in the tubes were 0.5 cubic centimeter of a two percent mouse red blood cell suspension.

The contents of the tubes were mixed then placed in the 37°C. water bath for one hour. The results of these titrations are recorded in table number 1.

TABLE NO. 1. TITRATION OF COMPLEMENT

Tube number	Complement serum 1:10	Saline (0.85%)	Antimouse Amboceptor (2 units)	Mouse corpuscles 2.0%	12/27/58	12/30/58	4/10/59	4/12/59	4/15/59	4/18/59
1	0.10	1.4	0.5	0.5	0	0	0	0	0	0
2	0.15	1.4	0.5	0.5	0	0	0	0	0	0
3	0.20	1.3	0.5	0.5	0	0	0	0	0	0
4	0.25	1.3	0.5	0.5	0	0	0	0	0	0
5	0.30	1.2	0.5	0.5	0	0	0	0	0	0
6	0.35	1.2	0.5	0.5	0	0	2	2	2	2
7	0.40	1.1	0.5	0.5	0	0	2	2	2	2
8	0.45	1.1	0.5	0.5	2	2	2	2	2	2
9	0.50	1.0	0.5	0.5	2	2	2	2	2	2
10	none	2.0	0.5	0.5	0	0	0	0	0	0

2- complete hemolysis

0 -no hemolysis

Tube number 10 represents a control for red blood cells and hemolysin and should show no hemolysis.

The tube with the least amount of complement serum which caused complete hemolysis was noted and the tube containing the next higher amount was taken as the full unit of complement. Twice this amount or two full units were used in the tests.

Frequent titration of complement is necessitated by the lability of the substance. Unless it is kept frozen, complement rapidly loses its power to bind the components of the complement fixation reaction. Unless in use, the complement for these experiments was kept refrigerated at -72°C. Each fresh vial of complement was titrated to insure against any error which may have been introduced because of a different origin of the substance or of its having been prepared under differing conditions.

Hemolytic amboceptor was prepared by a series of intravenous injections of washed mouse red blood cells into a rabbit. By heating this rabbit serum at 56°C. for 30 minutes the heat labile complement was destroyed. In order to insure adequate adjustment of the hemolytic system a titration was made between the hemolytic amboceptor and the complement (table number 2). Dilutions of the amboceptor, varying from 1:100 to 1:600, were employed in 0.5 cubic centimeter quantities. To each of these tubes was added 0.8 cubic centimeter of a 1:10 dilution of complement and 0.5 cubic centimeter of a two percent suspension of mouse red blood cells. The contents of each tube were then made up to a total volume of 3.5 cubic centimeter with the addition of 1.7 cubic centimeters of saline. The tubes were shaken gently and placed in the

TABLE NO. 2. TITRATION OF ANTIMOUSE AMBOCEPTOR

Tube number	Antimouse amboceptor (0.5cc)	Complement serum 1:10	Mouse corpuscles 2.0%	Saline (0.85%)	12/27/58	12/30/58	4/10/59	4/12/59	4/15/59	4/18/59
1	1:100	0.8	0.5	1.7	2	2	2	2	2	2
2	1:200	0.8	0.5	1.7	2	2	2	2	2	2
3	1:300	0.8	0.5	1.7	0	0	0	0	0	0
4	1:400	0.8	0.5	1.7	0	0	0	0	0	0
5	1:600	0.8	0.5	1.7	0	0	0	0	0	0
6	—	—	0.5	2.5	0	0	0	0	0	0

2- complete hemolysis

0- no hemolysis

37°C. water bath for one hour before reading. The highest dilution of amboceptor which in dose of 0.5 cubic centimeter caused complete hemolysis was the amboceptor unit. Two units were employed in the various titrations and the test itself. The two units were contained in 0.5 cubic centimeter of a dilution twice as strong as that which gave the unit.

The endpoint of this titration remained stable over the five month period in which titrations were made. This indicated that storage of the amboceptor -72°C. was quite adequate to preserve its integrity as a sensitizing agent for mouse red blood cells.

Todd and Sanford (21) state that the one indispensible property of an antigen is its "antigenic power". This refers to the ability of antigen to unite with or "fix" complement in the presence of an antiserum specific for it. Antigens may demonstrate two objectionable characteristics. They may exhibit anticomplementary properties which are characterized by an antigenic absorption of complement in spite of the fact that the antiserum is not present. Antigens may hemolyze the red blood cells used as an indicator mechanism of the complement-fixation test. The antigenic properties of an antigen are independent of their anticomplementary and hemolytic properties. The latter two are usually much weaker then the former; therefore, if a strong antigen is used the amount of error introduced into the test is negligible.

The antigens employed in the cross reaction series of tests were prepared by two methods. The first consisted of suspending one gram quantities of abscess material, taken directly from a liver infected with S. necrophorus, in one cubic centimeter of saline solution. This

paste was used in the preparation of antigen dilutions. The second type of antigen consisted of 48 hour cultures of the organism which were grown at 37°C. in either brain-heart infusion medium (Difco) or in fluid thioglycollate medium (Difco). The organisms were killed by heating the cultures to 60°C. for 30 minutes.

These two types of antigen were employed to determine whether the organisms derived from an in vitro source provided a better antigen than those developed in vivo.

The antigens employed in the determination of the cell wall and somatic antigenicity of the organism, were prepared in the following manner. A 1000 cubic centimeter flask was filled with brain-heart infusion broth and sterilized. The medium was cooled and inoculated with 10 cubic centimeters of a 24 hour culture of S. necrophorus strain 11366. The medium was then sealed in the flask with a paraffin plug. The culture was allowed to incubate at 37°C. for 3 days. At the end of the incubation period the wax plug had been forced up the neck of the flask by gas production. A dark grey layer of soft mucoid growth was observed on the sides and bottom of the flask. The plug was removed and a gram stain was made to confirm the purity of the culture. The medium was centrifuged in an International Centrifuge at 2000 times gravity for 20 minutes. The supernatant fluid was discarded. The organisms were washed twice with saline solution to insure the removal of extraneous matter. Ten cubic centimeters of distilled water were added to the organisms and lysis was enhanced by rapid stirring of the organisms in this water. The cell wall component was separated from the somatic

component by centrifugation at 2000 times gravity for 30 minutes. The supernatant, it was assumed, contained the somatic antigen components and the precipitate the cell wall antigens.

The titration of antigen for antigenicity was carried out using antigen dilutions between 1:300 to 1:2400. One-half cubic centimeter volumes of antigen were used. The antigens were placed in conjunction with 0.5 cubic centimeter quantities of a 1:80 dilution of inactivated S. necrophorus serum (11366). The inactivation was effected by heating the serum at 56°C. for 15 minutes. Inactivation refers to the destruction of the complement fraction of the serum. Although only serum 11366, rather then a pool of several strongly positive antisera, was employed in this titration it seemed to provide suitable endpoints in the titration of antigen. One-half cubic centimeter of a 1:5 dilution of complement was also placed in each tube. The tubes were placed in a 37°C. water bath for five minutes and refrigerated at 6°C. for 15 hours. A 1:100 dilution of antimouse amboceptor and a two percent mouse red blood cell suspension, each in the amount of 0.5 cubic centimeter, was placed in each of the tubes after their removal from the refrigerator.

The tubes were placed in a 37°C. water bath for one hour and read. The results of the titration are recorded in table number 3. Of special interest in this titration was the fact that with serum 11366 all of the antigens, in dilutions ranging from 1:1600 to as high as 1:2400, bound complement. Tube 11 was the serum control and tube 12

the control for the hemolytic system. Both of these controls should at all times demonstrate complete hemolysis.

The highest dilution which caused complete inhibition is the antigenic unit. The dose, used in these complement-fixation tests, consists of ten antigenic units and these were contained in 0.5 cubic centimeter of a dilution ten times as strong as that which gave the unit. This must be twenty times less then the anticomplementary or hemolytic unit. The results of the titration for antigenicity is shown in table 3.

The anticomplementary titration of antigen was conducted as illustrated in table number 4. Into each of ten tubes was placed 0.5 cubic centimeter of antigen in dilutions ranging from 1:4 to 1:40. To each of these tubes was added 0.5 cubic centimeter of inactivated normal bovine serum which had previously been diluted 1:80 with saline solution. Into each tube was placed 0.8 cubic centimeter of a 1:10 dilution of complement. This dilution and quantity of complement, equal to two units, was determined in the complement titration. The contents of the tubes were mixed well then placed in a water bath at 37°C. for five minutes. The water bath was used to facilitate the union of the components. The tubes were then placed in a refrigerator and cooled at 6°C. for 15 hours. It is believed that by allowing the components to come into conjunction in a cool environment a more specific union would be made between them. Following refrigeration 0.5 cubic centimeter of a 1:100 dilution of antimouse amboceptor, which by the titration of table number 2 was found to be equivalent to two amboceptor units, was added to each tube. To each tube was added 0.5 cubic centimeter of

TABLE NO. 3. TITRATION OF ANTIGEN FOR ANTIGENIC PROPERTIES

Tube number	Antigen dilution 0.5 cc	Inactivated S.n. serum (11366) 1:80	Complement 1:5	Antimouse amboceptor 2 units	Mouse corpuscles 2.0%	"L-abscess"	"L-thio."	"Q-abscess"	11366 BHI	M-2 BHI	"E-abscess"	"P-abscess"	11366 BHI and "P-abscess"	Somatic	Cell wall
													11366		
1	1:300	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
2	1:400	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
3	1:500	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
4	1:600	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
5	1:800	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
6	1:1000	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
7	1:1200	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
8	1:1600	0.5	0.5	0.5	0.5	0	0	0	0	0	0	0	0	0	0
9	1:1200	0.5	0.5	0.5	0.5	2	0	2	0	0	2	2	0	2	0
10	1:2400	0.5	0.5	0.5	0.5	2	2	2	2	2	2	2	2	2	0
11	0.5cc saline	0.5	0.5	0.5	0.5	2	2	2	2	2	2	2	2	2	2
12	1.0cc saline	none	0.5	0.5	0.5	2	2	2	2	2	2	2	2	2	2

2- complete hemolysis
0- no hemolysis

a two percent suspension of mouse red blood cells. Also present in this titration were a serum control and a hemolytic system control. These were prepared as shown in table number 4. Following the addition of all components the tubes were placed in the water bath at 37°C. for one hour and read.

The hemolytic power of the antigen was titrated in the following way. Dilutions of antigen varying in strength from 1:4 to 1:32 were placed in each of ten test tubes in the amount of 0.5 cubic centimeter. To each of these tubes was added 2.0 cubic centimeters of a 0.85 percent saline solution and a two percent suspension of mouse red blood cells. The tubes were placed in the water bath at 37°C. for one hour. None of the antigens were found to be hemolytic in nature.

The titrations of the anticomplementary and hemolytic properties of antigen demonstrated that all of the antigens employed in these experiments were of a suitable nature to be used in further tests.

The antisera employed in these tests were heated in a water bath at 56°C. for 30 minutes to destroy the heat-labile complement. Some antisera have been demonstrated to possess the capacity to hemolyze the red blood cells of the indicator system of this reaction. In an effort to determine whether or not any of the antisera used in these experiments were hemolytic in nature the titration illustrated on page 19 was set up.

Into each of seven tubes was placed 0.5 cubic centimeter of each of the respective antisera in dilutions ranging from 1:10 to 1:640. To each tube was added 0.8 cubic centimeter of a 1:10 (two full units)

TABLE NO. 4. TITRATION OF ANTIGEN FOR ANTICOMPLEMENTARY PROPERTIES.

Tube number	Antigen dilution 0.5 cc	Inactivated normal serum 1:80	Complement 1:10	Antimouse amboceptor 2 units	Mouse corpuscles 2.0%	"L-abscess"	"L-thio."	"O-abscess"	11366 BHI	M-2 BHI	"E-abscess"	"P-abscess"	11366 BHI and "P-abscess"	11366 Somatic	11366 Cell wall
1	1:4	0.5	0.8	0.5	0.5	0	0	0	0	0	0	0	0	0	0
2	1:5	0.5	0.8	0.5	0.5	0	0	0	0	0	0	0	0	0	0
3	1:6	0.5	0.8	0.5	0.5	0	2	0	2	2	0	0	0	2	0
4	1:8	0.5	0.8	0.5	0.5	0	2	0	2	2	0	2	2	2	0
5	1:10	0.5	0.8	0.5	0.5	0	2	0	2	2	2	2	2	2	0
6	1:12	0.5	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2
7	1:16	0.5	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2
8	1:20	0.5	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2
9	1:24	0.5	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2
10	1:40	0.5	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2
11	0.5 cc saline	0.5	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2
12	0.5 cc saline	none	0.8	0.5	0.5	2	2	2	2	2	2	2	2	2	2

2- complete hemolysis
0- no hemolysis

dilution of complement. Two cubic centimeters of 0.85 percent saline solution were added to each tube as was 0.5 cubic centimeter of a two percent suspension of mouse red blood cells. Tube number eight represents a red blood cell control. The contents of the tubes were mixed, placed in the 37°C. water bath for one hour, and read. (See table number 5)

From the data compiled in the above titration it became evident that all of the antisera tested possessed, at least in the lowest dilution, some degree of hemolytic ability. It was concluded, however, that the hemolytic effect of the antisera would be diminished after placing them in conjunction with both the antigen and complement prior to their exposure to the red blood cells of the hemolytic system.

The suspensions of red blood cells were prepared by withdrawing blood, by means of a 27 gauge needle and a syringe, from the chambers of the hearts of mice. The blood was placed in a sodium citrate solution to prevent it's clotting. The cells were spun down in a Servall angle centrifuge. By means of a capillary pipette and bulb the "buff coat" of leucocytes, which had sedimented on top of the red blood cells, was removed. These cells were removed because it was believed that they would interfer with the complete union of the components of the hemolytic system, i.e., the amboceptor, complement, and red blood cells. The packed red cells were diluted with saline to a concentration of two percent.

The test proper was set up in the following manner. Into each of five tubes was placed a 1:200 or 1:240 dilution of antigen. Into each tube was then placed 0.1, 0.05, 0.025, 0.005, and 0.0025 cubic

TABLE NO. 5. TITRATION FOR HEMOLYTIC NATURE OF ANTISERUM

Tube number	Antiserum dilution 0.5 cc	Complement (1:10)	Saline (0.85%)	Mouse corpuscles 2.0%	Antisera																Normal
					"L"	"C"	M-2	11366	"D"	"E"	"G"	"H"	"I"	"J"	"K"	"A"	"M"	"N"	"O"	"P"	
1	1:10	0.8	2	0.5	2	2	2	2	2	2	2	2	1	2	2	1	2	2	2	2	1
2	1:20	0.8	2	0.5	2	1	2	1	2	2	0	2	1	1	2	0	2	2	1	0	0
3	1:40	0.8	2	0.5	0	0	1	1	1	1	0	2	0	0	2	0	0	2	0	0	0
4	1:80	0.8	2	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
5	1:160	0.8	2	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	1:320	0.8	2	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	1:640	0.8	2	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	none	none	2	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

2- hemolysis

1- partial hemolysis

0- no hemolysis

centimeters of a 1:5 dilution of complement. Each 0.5 cubic centimeter volume employed bore two units of complement as was determined in the titration illustrated in table number 1. The tubes were gently shaken and placed in the 37°C. water bath for five minutes and refrigerated at 6°C. for fifteen hours. Following refrigeration, the tubes were again placed in the 37°C. water bath for five minutes so that the components, already in conjunction in the tubes, might receive the hemolytic agents more readily. Two units of antimouse amboceptor, in the amount of 0.5 cubic centimeter, were placed in each tube. The amboceptor unit was determined as depicted in table number 2. To each of these tubes was then added 0.5 cubic centimeter of a two percent suspension of mouse red blood cells. The contents of the tubes were mixed and incubated in a water bath for one hour then read. In each of the tests performed an antigen, hemolytic, and corpuscle control was used. The antigen control consisted of antigen, complement, red blood cells and amboceptor. The hemolytic control was made up of complement, amboceptor and red blood cells. The red blood cell control was prepared by simply suspending red blood cells in saline solution. In all of the tests both the antigen and hemolytic control exhibited complete hemolysis and the red blood cell control showed no hemolysis. These were the results expected in these controls therefore no error was believed to have been introduced by the components involved in these controls. The results of the tests are recorded in table number 6.

All of the antigens except for the "L-abscess" antigen reacted,

centimeters of a 1:5 dilution of complement. Each 0.5 cubic centimeter volume employed bore two units of complement as was determined in the titration illustrated in table number 1. The tubes were gently shaken and placed in the 37°C. water bath for five minutes and refrigerated at 6°C. for fifteen hours. Following refrigeration, the tubes were again placed in the 37°C. water bath for five minutes so that the components, already in conjunction in the tubes, might receive the hemolytic agents more readily. Two units of antimouse amboceptor, in the amount of 0.5 cubic centimeter, were placed in each tube. The amboceptor unit was determined as depicted in table number 2. To each of these tubes was then added 0.5 cubic centimeter of a two percent suspension of mouse red blood cells. The contents of the tubes were mixed and incubated in a water bath for one hour then read. In each of the tests performed an antigen, hemolytic, and corpuscle control was used. The antigen control consisted of antigen, complement, red blood cells and amboceptor. The hemolytic control was made up of complement, amboceptor and red blood cells. The red blood cell control was prepared by simply suspending red blood cells in saline solution. In all of the tests both the antigen and hemolytic control exhibited complete hemolysis and the red blood cell control showed no hemolysis. These were the results expected in these controls therefore no error was believed to have been introduced by the components involved in these controls. The results of the tests are recorded in table number 6.

All of the antigens except for the "L-abscess" antigen reacted,

TABLE NO. 6. RELATIONSHIP OF STRENGTH OF IN VIVO WITH IN VITRO ANTIGENS

Antigen	Antisera: "L"	"C"	M-2	11366	"D"	"E"	"G"	"H"	"I"	"J"	"K"	"A"	"M"	" N"	"O"	"P"	Normal	No. tubes/titer: Very strongly pos.	Strongly pos.	Moderately pos.	Weakly pos.	Negative
"L-abscess"	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	—	—	—	—	17
"O-abscess	0	2	0	1	4	0	0	0	0	0	0	0	0	0	4	0	1	2	—	1	2	12
"E-abscess"	1	3	2	2	0	3	4	4	3	3	4	4	0	4	3	3	2	5	6	3	1	2
"P-abscess"	2	2	0	0	2	2	2	2	3	3	2	3	2	2	3	3	1	—	5	9	1	2
														Average				1.75	2.75	3.25	1.0	8.25
														Percent				10.3	16.2	19.1	5.9	48.5
"L-thio."	3	3	2	2	0	2	2	0	0	3	0	0	0	0	0	0	0	—	3	4	—	10
11366 BHI	0	2	4	3	3	4	4	0	0	4	0	0	0	0	4	1	4	6	2	1	1	7
M-2 BHI	0	2	3	2	0	3	1	0	0	3	0	0	1	1	0	1	1	—	3	2	5	7
														Average				2.00	2.66	2.33	2.00	8.00
														Percent				11.8	15.6	13.7	11.8	47.1

Partial or complete inhibition in first four tubes-very strongly positive-4-
Partial or complete inhibition in first three tubes-strongly positive-----3-
Partial or complete inhibition in first two tubes-moderately positive-----2-
Partial or complete inhibition in first tube-weakly positive----------1-
Complete hemolysis in all tubes-negative----------0-

at least to some degree, with the antisera tested.

In an attempt to compare the degree of antigenicity of in vivo with antigens produced in vitro, the antibody titer of each reaching antiserum was recorded. The total number of tubes of each antibody titer was tabulated for each antigen. The sum of the number of tubes of each titer for the in vivo antigens and for the in vitro antigens was then averaged with the total number of antigens employed either as in vivo or in vitro antigens. This average was then divided by seventeen (the number of antisera employed) to give the percentage of reactions occuring at each titer. (See table number 6)

Partial or complete inhibition in the first four tubes was recorded in 10.3 percent of the in vivo antigens and 11.8 percent of the in vitro antigens. Partial or complete inhibition in the first three tubes was demonstrated in 16.2 percent of the in vivo and 15.6 percent of them in vitro antigens. Nineteen and one-tenth percent of the in vivo and 13.7 percent of the in vitro antigens showed partial or complete inhibition in the first two tubes. Partial or complete inhibition was demonstrated in the first tube of 5.9 percent of the in vivo and 11.8 percent of the in vitro antigens. A negative reaction was recorded in 48.5 percent of the in vivo and 47.1 percent of the in vitro antigens. By this comparision it was shown that with the exception of the third (moderately positive) and the fourth (weakly positive) titers listed above, the reaction types of the in vivo and in vitro antigens varied by not more than 1.5 percent. No explanation can be offered for the weakly positive results appearing in the reactions with

the normal bovine serum.

A peculiar characteristic brought out by the "E-abscess" antigen and the 11366 BHI antigen, concerned the demonstration, by antisera not specific for the antigens, of an antibody titer higher than that of the antisera specific for those antigens.

Using the cell wall as the antigen source, no endpoint was reached in an antigen dilution to 1:2400. The somatic material of the cells demonstrated an endpoint at a dilution of 1:1200. (See table number 3) It may be tentatively concluded from this experiment that although the cell wall provides the main source of antigenic stimulus the somatic material also exhibits a degree of antigenicity. The antigenicity attributed to the somatic portion of the cell may be partially due to water soluble surface components of the lysed cells.

An attempt was made to develop an antigen common to all of the antisera. Two antigens were chosen which together should have given a positive reaction with all of the antisera. 11366 BHI and "P-abscess" were selected as these sources of antigen. Each of these antigens was diluted to contain ten units per half cubic centimeter. These two antigens sources were then pooled and used in 0.5 cubic centimeter aliquots as a single antigen. The remainder of the test was performed as described previously.

Table number 7 shows that the combined antigens seem to complement each other. The combined antigen, in all cases, demonstrated a greater

TABLE NO. 7. DERIVATION OF A COMMON ANTIGEN

Antigen	Antisera																
	"L"	"C"	M-2	11366	"D"	"E"	"G"	"H"	"I"	"J"	K"	"A"	"M"	"N"	"O"	"P"	Normal
"P-abscess"	2	2	0	0	2	2	2	2	3	3	2	3	2	2	3	3	1
11366 BHI	0	2	4	3	3	4	4	0	0	4	0	0	0	0	4	1	4
"P-abscess" plus 11366 BHI	1	3	2	3	3	2	4	2	4	4	3	4	3	2	4	1	1

Partial or complete inhibition in first four tubes-very strongly pos.-4

Partial or complete inhibition in first three tubes-strongly pos.-----3

Partial or complete inhibition in first two tubes-moderately pos.-----2

Partial or complete inhibition in first tube-weakly pos.----------1

Complete hemolysis in all tubes-negative----------0

effectiveness in uniting with complement and antisera, than either of the two individual components of which it was composed.

Shaw (18) was of the opinion that no method of diagnosis of S. necrophorus, by agglutination tests, was feasible. This work, although not utilizing a sufficient number of antisera to make the results conclusive, supports the theory that a modification of the complement-fixation reaction might successfully be used in the diagnosis of S. necrophorus infection.

Most workers (5, 16) are of the opinion that the organism is extremely diverse in its antigenicity. This conclusion, in almost all cases, is based upon the results of agglutination reactions. The results of these complement-fixation reactions support the concept that S. necrophorus antigens, derived principally from bovine sources, are very homogenous in nature. It seems apparent that the complement effects a broader, more complete union between antigen and antiserum, than can be demonstrated by the use of antigenic agglutinins.

SUMMARY AND CONCLUSIONS

This study was undertaken in an attempt to gain a further insight into the serological activity of Sphaerophorus necrophorus. Little serological work has been performed with this organism and no literature could be found dealing with complement fixation reactions of S. necrophorus.

1.) All of the antigens employed were non-hemolytic in nature.

2.) All of the antisera were hemolytic to a small degree.

3.) A temperature of -72°C. was adequate to preserve the integrity of both complement and amboceptor.

4.) Some normal antisera demonstrated a low antibody titer.

5.) For all practical purpose in vivo antigens were almost identical to in vitro antigens in their response to antisera in the complement fixation test.

6.) Almost 50 percent of the single antigen, antisera reactions failed to produce positive results.

7.) Two antigens were chosen which when pooled reacted positively with the entire spectrum of antisera employed in these tests. Thereby a common antigen was demonstrated for all of the serum samples.

8.) The pooled antigens of the common antigen seemed to complement each other for together they reacted to a greater degree then each did individually.

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